Repeated Remote Ischemic Postconditioning Protects Against Adverse Left Ventricular Remodeling and Improves Survival in a Rat Model of Myocardial Infarction

Meng Wei, MD, PhD,* Ping Xin, MD, PhD,* Shuai Li, MD, Jianping Tao, MD, Yapeng Li, MD, Jing Li, MD, PhD, Mingya Liu, MD, PhD, Jingbo Li, MD, PhD, Wei Zhu, MD, Andrew N. Redington, MD

Rationale: Remote ischemic conditioning induced by repeated episodes of transient limb ischemia is a clinically applicable method for protecting the heart against injury at the time of reperfusion. Objective: To assess the effect of chronic, repeated, remote conditioning on infarct size and long-term remodeling after myocardial infarction.

Methods and Results: Rats with ischemia/reperfusion injury received different protocols of remote limb conditioning. While a single early episode of remote ischemic conditioning during coronary occlusion (perconditioning) resulted in a decrease in infarct size on both day 4 and day 28, when it was repeated (postconditioning) intermittently (every 3 days) and intensively (every day), it was not associated with a further decrease in infarct size. However, the protection against adverse remodeling offered by a single episode of limb perconditioning was further enhanced by repeated remote postconditioning therapy in a dose-dependent manner. In separate experiments there was a dose-dependent improvement in survival at 84 days by Kaplan–Meier analysis.

Conclusions: Whereas a single early episode of remote perconditioning reduces infarct size, repeated remote postconditioning further reduces adverse LV remodeling and improves survival in a dose-dependent fashion. These data may have clinical implications for the treatment of patients with evolving myocardial infarction. (Circ Res. 2011;108:00-00.)

Key Words: heart failure • remote postconditioning • myocardial infarction • remodeling • reperfusion

Despite the timely application of reperfusion therapy, survivors of acute myocardial infarction (MI) are at significant risk from later myocardial remodeling, leading to the development of heart failure and elevated risk of death during 5-year follow-up. It is generally agreed that the acute inflammatory process that occurs early after acute MI (a prerequisite for adequate healing and scar formation) can be adverse if persistent in the later post-MI recovery period. Continued oxidative stress results in proinflammatory cytokine release, cardiomyocyte apoptosis, ventricular fibrosis, and hypertrophy.

Remote ischemic conditioning reduces infarct size when applied both before (preconditioning) and during (perconditioning) experimental and clinical MI. Similarly, local postconditioning can reduce infarct size. Clinically, postconditioning is achieved by repeated inflation and deflation of the angioplasty balloon after emergency coronary intervention. It is now known that postconditioning can be induced experimentally and in humans, remotely, using a transient ischemia stimulus identical to that of remote pre- and preconditioning, and in a recent study, remote postconditioning was more effective than local postconditioning in experimental MI. We have shown that repeated daily remote conditioning by limb ischemia leads to significant downregulation of neutrophil activation and proinflammatory responses in humans. Thus, in the present study we investigated (1) whether a single episode of remote perconditioning protects against late remodeling; (2) whether a further decrease in infarct size or improved remodeling can be achieved when chronic remote postconditioning is given in addition to early preconditioning; and (3) how pathological processes involved in remodeling—including oxidative stress, inflammatory responses, and fibrotic and hypertrophic signaling—are modulated by repeated remote postconditioning.
Methods
Details of the study groups, experiment protocol, infarct size quantification, hemodynamic evaluation, and analytic methods are provided in the Online Data Supplement at http://circres.ahajournals.org. In brief, we established myocardial infarction by 45 minutes of LAD ligation, followed by reperfusion (MI group). Additional groups of animals (Figure 1A) were subjected to a single episode of remote perconditioning during ischemia (4 cycles of 5 minutes of hindlimb ischemia, 5 minutes of reperfusion, PerC group), perconditioning plus postconditioning (4 cycles of 5 minutes of hindlimb ischemia, 5 minutes of reperfusion) every 3 days for 28 days (D-3PostC group), and perconditioning plus postconditioning every day for 28 days (D-1PostC group). Different groups of animals were euthanized at 7 and 28 days and comparison was made with a sham-operated group, and a group subjected to daily sodium pentobarbital anesthesia (to control for daily intervention, SP group). In separate experiments, Kaplan–Meier survival was assessed in the same intervention groups (50 animals per group).

Results
Details are provided in the Online Data Supplement at http://circres.ahajournals.org.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-1PostC</td>
<td>daily remote ischemic postconditioning for 28 days</td>
</tr>
<tr>
<td>D-3PostC</td>
<td>remote ischemic postconditioning every 3 days for 28 days</td>
</tr>
<tr>
<td>PerC</td>
<td>remote ischemic preconditioning</td>
</tr>
</tbody>
</table>

Improved Survival
PerC, D-3PostC, and D-1PostC resulted in improved survival rate (Figure 1B) in comparison with MI and SP groups ($P<0.05$, for all). Interestingly, the improved survival was apparent as early as 28 days after MI only in the D-1PostC group, whereas in the D-3PostC group this effect was not observed until 56 days after MI. Furthermore, on day 84, D-1PostC was associated with improved survival in comparison with PerC and D-3PostC treatments ($P<0.05$, respectively).

Reduced Infarct Size
Infarct size was quantified for the 6 groups at 72 hours and at 28 days, with a similar pattern of response at each time point (Figure 2A through 2D). There was a reduction in infarct size, resulting in all conditioning groups in comparison with MI ($P<0.05$, respectively), but no difference between the conditioning groups ($P>0.05$, respectively).

Attenuated Inflammatory Responses (Figure 3A through 3D)
There was intensive infiltration by both macrophages and neutrophils detected in the MI group, which was attenuated in PerC rats ($P<0.01$), and further attenuated by D-3PostC and D-1PostC ($P<0.01$ versus MI group, respectively) with the greatest effect detected in D-1PostC group rats ($P<0.05$ versus D-3PostC group). The expression level of MCP-1 in the infarction border zone on day 4 showed the same pattern,

Figure 1. A, Experimental protocol. Rats were randomized to 6 different groups: (1) sham group, in which rats underwent sham operation without any intervention; (2) MI group, in which rats underwent 45 minutes LAD ischemia followed by reperfusion with no conditioning therapy; (3) remote perconditioning (PerC) group; (4) D-3PostC group, in which remote postconditioning was administered every 3 days; (5) D-1PostC group, in which remote postconditioning was administered every day for 28 days; and (6) SP group, in which MI rats were given sodium pentobarbital every day for 28 days to control for repeated anesthesia. Vertical arrows indicate the times for euthanization. B, Shows the Kaplan–Meier survival curves created for 6 groups treated identically but followed for 84 days. *$P<0.05$ versus MI group; **$P<0.001$ versus MI group; ***$P<0.05$ versus PerC group.
demonstrating a dose-dependent effect in the remote conditioning groups (P<0.05, respectively) (Figure 3E).

**Oxidative Stress, NF-κB Activation, and Cytokine Expression**

The concentration of MDA, and phosphorylation of the NF-κB subunit p65 and its inhibitory protein IκBα, was significantly increased in MI rats on day 28, and was significantly reduced by per- and postconditioning in a dose-dependent fashion (Online Figure IA through IC). Interestingly, on day 28, PerC did not modify TNF-α and IL-1β levels (P>0.05, respectively), which were only attenuated by both D-3PostC and D-1PostC groups (P<0.01 vs MI group, respectively), however, to the same degree (P>0.05) (Online Figure ID and IE).

**Fibrosis Responses**

The reduction of TGF-β1/Smad2 signaling activation in the infarct borderzone was consistent with the beneficial pattern of NF-κB signaling activation (Online Figure IIA and IIB), and further supported by attenuated interstitial fibrosis (Online Figure IIC through IIE). When TGF-β1/Smad2 signaling activation was quantified in remote heart tissue, D-1PostC also showed the greatest effect (Online Figure IIIA and IIIIB).

**Hypertrophic Responses**

There was increased LV mass in the MI group, which was attenuated by PerC (P<0.05), with a further decrease with both D-3PostC and D-1PostC (P<0.05 versus PerC group, respectively), with the lowest mass observed in D-1PostC (P<0.001, versus D-3PostC group). Hypertrophy-related gene expression of ANP and β-MHC was attenuated, whereas the decrease in expression of α-MHC was recovered by remote conditioning, again in a dosage-dependent fashion (Online Figure IVA through IVC).

**Improved Cardiac Geometry, Function, and Hemodynamics**

On day 28, MI rats demonstrated significant LV dilation and decreased fractional shortening in comparison with sham rats (both P<0.05). Although PerC significantly improved LV remodeling in comparison with MI rats (P<0.05, respectively), repeated remote PostC therapy resulted in a further improvement in LV chamber size and function, with the greatest effects achieved by D-1PostC in comparison with D-3PostC (P<0.05, respectively) (Figure 4A through 4D). Hemodynamic analysis demonstrated the same dose-dependent pattern of improvement (Online Figure VA through VC).

**Discussion**

This is the first study to demonstrate that remote ischemic conditioning not only reduces infarct size but improves late LV remodeling and survival after MI. Our data show that a single episode of remote perconditioning affords some long-term protection, but repeated remote postconditioning during the 28 days post-MI significantly improves adverse LV remodeling and function and survival in dose-dependent fashion, and was closely associated with attenuated myocardial inflammatory responses and oxidative stress.

**Role of Chronic Remote Postconditioning**

Since the phenomenon of ischemic preconditioning was first described by Murry et al., many studies have been performed to elucidate the mechanisms by which pre- and postconditioning induce cardioprotection. Although the exact...
mechanisms are not fully understood, it is generally agreed that the attenuation of reactive oxygen species (ROS) generation\textsuperscript{12–14} is of paramount importance in the protection afforded by these strategies. Furthermore, it is well established that continued ROS generation and inflammation is pivotal in the process of post-MI remodeling\textsuperscript{15,16}. Remote conditioning has additional effects on circulating monocytes, downregulating white cell proinflammatory pathways\textsuperscript{17}, and when delivered daily for 10 days, reducing neutrophil adhesion, phagocytosis, and proinflammatory cytokine responses\textsuperscript{10}. Our findings are consistent with reduced myocardial oxidative stress (and hence reduced NF-κB phosphorylation), decreased inflammatory cell migration into the infarct border zone (observed directly as attenuated MPO and ED-1 immunostaining density), and reduced local inflammatory cytokine signaling (reduced tissue MCP-1 expression, reduced circulating TNF-α and IL-1β levels). Local chemokines such as MCP-1 are responsible for inducing recruitment of mononuclear cells. Moreover, activated NF-κB pathways can also up-regulate the target gene expression of TNF-α and IL-1β.\textsuperscript{18} Although this modification of the local and circulating inflammatory milieu is likely crucial to the beneficial effects of chronic remote conditioning, more studies are required to dissect the mechanisms and assess whether it might be an adjunct to, or replace, current antiremodeling drug regimes frequently used after MI. Nonetheless, these findings have obvious relevance to post-MI recovery in humans. In this regard, and similar to other remote conditioning protocols, there is capacity for our observations to translate rapidly to clinical trials.\textsuperscript{19}

Limitations of the Study
We observed that, on day 28, PerC rats did not show a decrease in TNF-α and IL-1β expression, whereas both D-3PostC and D-1PostC showed a significant decrease in chemokine levels. This could reflect a dosage-dependent response; however, we also observed a decrease in NF-κB.
phosphorylation in the PerC group. Consequently, it is difficult to explain the apparent conflicting data on the basis of our data.

Although the data of improved survival rate were clear, we did not include any assessment of remodeling after cessation of repeated PostC. It is possible that the time course of adverse remodeling was simply delayed, rather than permanently modified.

Conclusion

We have shown that chronic remote ischemic postconditioning provides dose-dependent protection against pathological ventricular remodeling, and improves survival after myocardial infarction.

Sources of Funding

The study was supported by the Canadian Institutes for Health Research and the Leducq Foundation (to A.N.R.), by institutional financial support of Shanghai Jiao Tong University Sixth Hospital (to W.Z.), and partly by the Natural Science Foundation of China (No. 81070110; to M.W.).

Disclosures

None.

References


Figure 4. Representative M-mode echocardiograms for each group (A), with LVESD (B), LVEDD (C), and FS (D) quantified in bar graphs. Data are expressed as mean ± SD.

**Novelty and Significance**

**What Is Known?**
- Remote ischemic preconditioning induced by transient limb ischemia reduces myocardial infarct (MI) size in animals and humans.
- Remote ischemic preconditioning reduces proinflammatory neutrophil gene expression, and when repeated, daily postconditioning reduces neutrophil adhesion and phagocytosis.

**What New Information Does This Article Contribute?**
- Remote postconditioning administered after MI does not lead to further reduction in infarct size.
- Remote postconditioning administered after myocardial infarction in rats reduces adverse left ventricular (LV) remodeling at 28 days in a dose-dependent fashion.
- Improved remodeling is associated with reduced peri-infarct inflammation and fibrosis.

While the beneficial early effects of remote ischemic conditioning (induced by 4 cycles of 5 minutes of transient limb ischemia followed by reperfusion) on MI are well established, its effect when administered repeatedly after MI (remote postconditioning) has not been examined. We show here, for the first time, that remote postconditioning administered every 3 days, or every day, for 28 days improves post-MI LV remodeling at 28 days, and survival at 12 weeks, in a dose-dependent fashion. This simple technique is readily applicable to humans after MI and may lead to significant benefits in post-MI morbidity and mortality.
Repeated Remote Ischemic Postconditioning Protects Against Adverse Left Ventricular Remodeling and Improves Survival in a Rat Model of Myocardial Infarction
Meng Wei, Ping Xin, Shuai Li, Jianping Tao, Yapeng Li, Jing Li, Mingya Liu, Jingbo Li, Wei Zhu and Andrew N. Redington

Circ Res. published online April 7, 2011;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2011/04/07/CIRCRESAHA.110.236190

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/04/07/CIRCRESAHA.110.236190.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplement Material

Methods:

Animals

Male Sprague-Dawly (SD) rats, weighing between 250g and 280g (Experimental Animal Center, Fudan University, Shanghai, P.R. China) were studied. The animal research study protocol was in compliance with ‘The Guide for the Care of Use of Laboratory Animals’ published by the National Institute of Health (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care Committee of Shanghai Sixth Hospital, Shanghai Jiao Tong University School of Medicine. All the rats were housed for two weeks as an acclimatization period before the experiments.

Surgical Preparation

After anesthesia with sodium pentobarbital (80 mg/kg, IP) and endotracheal intubation, animals were ventilated (Animal Respirator DW-2000, Aloctt Biotech, Shanghai, P. R. China) with room air. The heart was exposed through a thoracotomy at the left fourth intercostal space. The left anterior descending coronary artery (LAD) was encircled by a 6-0 prolene suture 1 to 2 mm below the tip of the left atrial appendage and its ends were threaded through a polyethylene tubing (PE-50) to form a snare for reversible coronary artery occlusion. Prior to LAD occlusion, the animals were anticoagulated (150U/Kg sodium heparin) and received an intravenous injection of lidocaine (4mg/Kg). Cardiac ischemia was confirmed by a pale area below the suture or ST-T elevation shown in ECG, while reperfusion was characterized by rapid disappearance
of cyanosis and vascular blush [1]. At the end of the protocol, the snare was removed, the chest closed, and the animals allowed to recover, and they were given one dose of tamgesic (0.03mg/kg intramuscularly) was given for pain relief immediately before they gained consciousness.

Experimental Protocol

After anesthesia, all rats were randomly assigned to the following six experimental groups: 1) MI group, MI was created with 45 mins LAD ligation followed by reperfusion for further observation; 2) remote ischemic perconditioning (PerC) group, during the ischemia reperfusion injury surgical procedure as in group 1, and while the animals were anesthetized, PerC was delivered starting 20mins before the end of index ischemic period by occluding hind limb blood flow with a torniquet tightened around the upper thigh for 4 cycles of 5 minutes occlusion followed by 5 minutes release. The limb ischemia was confirmed by pallor and cyanosis of the lower limb below the torniquet; 3) remote ischemic postconditioning delivered every 3rd day (D-3 PostC group), after the initial PerC maneuver, MI rats were anesthetized again with smaller dosage of sodium pentobarbital (30mg/Kg intraperitoneally) and received the remote postconditioning stimulus every 3rd days for 28 days with second treatment starting on day 4 (72 hours after reperfusion). The remote postconditioning was delivered in the same way and using an identical protocol as described above for PerC except that only sodium pentobarbital was used; 4) daily remote ischemic postconditioning (D-1 PostC group), the same remote postconditioning therapy as described above was
repeated every day for 28 days for MI rats; 5) sodium pentobarbital group (SP group), to act as a control for the effects of repeated anaesthesia, MI rats received sodium pentobarbital (30mg/Kg intraperitoneally) every day for 28 days after the surgical procedure to test the effects of repeated anesthesia; and 6) a sham group, rats underwent sham operation without suture tie-down of the LAD. All the surviving rats that completed the observation period were sacrificed either on day 4 (72 hours after reperfusion) or on day 28 after reperfusion, respectively for testing outlined below, and detailed in Figure I. All animal assigned to the D-3 PostC therapy group on either day 4 or day 28 were euthanized 2 hours after the episode of postconditioning. It should be noted that other than the single dose of temgesic for pain relief at the time of thoracotomy, no animal received any medication other than sodium pentobarbital anesthesia as per the protocols outlined above. Echocardiographic and invasive hemodynamic examination (described as below) was performed to evaluate the remodeling process immediately before euthanization on day 28.

After euthanization, whole blood was collected from inferior vena cava and the heart was harvested. LV weight was recorded and the ratio of LV weight to body weight was calculated to derive left ventricle mass index (LVMI) on day 28. One cross-section of LV myocardial tissue at the level of the papillary muscles, approximately 5mm, was collected and fixed in 4% formalin followed by paraffin embedding for the histology examination. The remaining LV tissue was quickly separated into peri-infarct zone and remote zone, and tissue were snap frozen in liquid nitrogen, and mechanically homogenized in liquid nitrogen and re-suspended in
lysis buffer where phosphatase was added. The tissue was then further lysed in a tightly fitting cylinder homogenizer. The sample was then stored at -80 degrees Celcius for later analysis.

To evaluate the survival rate, another 250 rats were assigned to MI group, PerC group, D-3 PostC group, D-1 PostC group and SP group (50 rats for each group) while 25 sham operated rats acted as controls. All the rats were rigorously monitored for 12 weeks. A careful autopsy was performed for each rat searching for the cause of sudden death, especially in reference to cardiac rupture. Survival rate was analyzed to evaluate the long term benefits of the treatments.

**Echocardiography and Hemodynamic Study**

Transthoracic echocardiography was performed on day 28 after MI using a standard ultrasound system equipped with a 15-MHz probe (Acuson Sequoia 512). Both two-dimensional and M-mode echocardiography was obtained after the induction of anesthesia. LV end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD) was measured in short axis view at papillary muscle level. The fractional shortening (FS) was also calculated. All the values were averaged over five consecutive cardiac cycles and measurements were analyzed by two independent researchers blinded to treatment protocol. Thereafter, cardiac catheterization was performed in animals for hemodynamic study. The right carotid artery was cannulated with a pre-heparinized fine polyethylene tube connected to a fluid-filled pressure transducer (MPA-CFS,
Alcott Biotech, Shanghai, China) and the tube was then advanced into the left ventricle. Heart rate (HR), left ventricular end-diastolic pressure (LVEDP) and the maximal rates of rise and fall in LV pressure \( \frac{dP}{dt_{\text{max}}} \) and \( \frac{dP}{dt_{\text{min}}} \), respectively) were recorded.

**Myocardial Infarct Size Measurement**

On day 4 (72 hours after reperfusion), the LAD was re-occluded and 1 ml 1% Evan’s blue was perfused into the aorta and coronary arteries. The heart was then isolated, perfused with PBS and sliced transversely in a plane perpendicular to the apical–basal axis into 5 mm thick sections. Heart sections were then weighed and incubated in 1% 2,3,5–triphenyltetrazolium chloride (TTC) (Sigma) for 5 to 10 min at 37 °C. The infarct area (pale), the area at risk (red), and the total LV area from each section were measured using Image J software. Infarct size was calculated and expressed as a percentage of the sum of infarct areas from all sections corrected by weight over the total LV tissue at risk area from all sections and multiplying by 100 \(^1\).

To measure infarct size 28 days after MI, the LV area was estimated using a slice obtained from the central part of the myocardium at the level of the papillary muscles. The infarct (expressed as fibrotic area) perimeter was measured and the size of MI was normalized to circumference of the whole LV using the following equation:

\[
\text{percentage infarct perimeter (IP)} = \frac{\text{circumference of infarct scar}}{[(\text{epicardium perimeter} + \text{endocardium perimeter})/2] \times 100 \%} \quad \text{[2]}
\]
Histological Analysis

Deparaffinized tissue sections were prepared for immunohistochemical staining. First antibody against myeloperoxidase (MPO, 1:50 dilution, Abcam) and against ED-1 (a specific marker for macrophage, 1:100 dilution, Chemicon) were used for neutrophil and macrophage infiltration quantification, using paraffin embedded heart tissue obtained from day 4. The number of inflammatory cells was counted in the border zone of infarcted heart. The border zone was defined using previously published criteria. The infarct edge was usually obvious from staining differences, and further delineated as the outer limit of an area of thinned and often bulging myocardium. This transitioned to clearly normal myocardium (remote area) within approximately 5 mm from the infarct edge. Histological examination of the borderzone was performed, and heart tissue (sectioned from endo to epicardium) obtained for western blotting etc, in the tissue 2-3 mm from the infarct edge, between the infarct edge and normal myocardium. Sections incubated with secondary antibody alone served as negative control. All sections were counterstained with hematoxylin.

The deparaffinized sections of heart slices obtained on day 28 were also stained with Sirius red (a collagen specific dye), to allow a clear discrimination between cardiomyocytes and collagen matrix. Collagen deposition in the border area was confirmed and quantified by Masson trichrome staining. Collagen volume fraction was analyzed by using Image J software and expressed as the average percentage collagen staining of 20 randomized high power fields.
**Western Blot**

Heart tissue obtained from both border and remote zones (see above) of LV was used for western blot analysis. Equal amounts of protein (50 μg) were separated on 8-16% Tris-glycine gel (Novex, Invitrogen, CA, USA) and transferred to a PVDF membrane. After blocking with 5% skim milk, primary antibody against phospho-IκB-α (Ser32) (1:1000 dilution, Cat# 9246, Cell Signaling Tech), IκB-α (1:1000 dilution, Cat# 9242, Cell Signaling Tech), phospho-NF-κB-P65 (1:1000 dilution, Cat# 3036, Cell Signaling Tech), NF-κB-P65 (1:1000 dilution, Cat# 3034, Cell Signaling Tech), and TGF-β1 (1:400 dilution, Cat# sc-52893, Santa Cruz), phospho-Smad2 (1:1000 dilution, Cat# 3101, Cell Signaling Tech), and Smad-2 (1:200 dilution, Cat# sc-6200, Santa Cruz) were incubated overnight at 4°C followed by incubation with horseradish peroxidase conjugated secondary antibody either anti-mouse or anti-rabbit. Immuno-reaction was finally visualized using an ECL detection Kit (Amersham, Beverly, MA, USA). All protein expression levels were adjusted by GAPDH intensity (Cell Signaling Tech, USA). Bands were quantified by densitometry using the software of Image J (version 1.41, NIH, USA). MCP-1 expression level was also measured at peri-infarct area using primary antibody against MCP-1 (1:200 dilution, Cat#sc-28876, Santa Cruz, USA).

**Real Time RT Polymerase Chain Reaction**

Total RNA was prepared from 150 mg of LV tissue at remote area using Trizol reagent (Invitrogen) followed by chloroform extraction and isopropanol precipitation.
Genomic DNA was eliminated by incubating with DNAase I (0.1 μl^{-1}, 37°C) for 30 min followed by acid phenol-chloroform extraction. RNA was quantified by spectrophotometric absorbance at 260nm, with its purity confirmed by A_{260}/A_{280} ratio and integrity evaluated by ethidium bromide staining on a denaturing agarose gel.

Total RNAs (2μg) were then reverse transcribed using oligo (dT) primer and Superscript II reverse transcriptase (RT, Invitrogen).

Real-time PCR quantification was performed starting with 12.5ng cDNA and both sense and antisense primer at 900nM concentration (Invitrogen) in final volume of 25μl, using SYBR green master mix (Applied Biosystem). Fluorescence was monitored and analyzed in a GeneAmp 7000 detection system instrument (Applied Biosystems). The PCR reactions were cycled 42 times by a three-step cycle procedure (denaturation 95 °C, 15 s; annealing 60 °C, 30 s; extension 72 °C, 30 s) following the initial stage (95 °C, 10 min). A ΔCt value was obtained to quantify the mRNA levels and normalized with an endogenous control (β-tubulin mRNA) for each sample. A relative quantification ΔΔCt method was used for comparison between groups. Oligonucleotide primers were designed using Primer Express software (Applied Biosystems).

The primers used were listed as follows:

α-MHC, sense (CTGCTGGAGAGGTTATTCCTCG) and antisense (GGAAGAGTGAGCGGCGCATCAAGG);

β-MHC, sense (TGCAAAGGCTCCAGGTCTGAGGGC) and antisense (GCCAACACCAACCTGTCCAAGTTC);
ANP, sense (CTCTGAGAGACGGCAGTGCT) and antisense (TATGCAGATGGGAGAGGCA); 
β-tubulin, sense (TCACTGTGCCTGAACCTTACC) and antisense (GGAACATAGCCGTAATCG).

**Determination of Malondialdehyde (MDA) Level**

Myocardium obtained from the border zone on day 28 was homogenized in 1.0ml of 20mmol/L Tris-HCl, pH 7.4, containing 5 mmol/L butylated hydroxytoluene. Lipid peroxides were assayed using a commercial available kit (Cat# 437639, Calbiochem) according to the manufacturer’s instructions, and level of MDA was expressed as μM per gram protein.

**Cytokine Levels**

Plasma levels of interleukin TNF-α and IL-1β were evaluated by use of commercially available solid-phase sandwich ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer’s introduction. The detection limits of each assay were as follows: TNF-α, 16 pg/ml and IL-1β, 10 pg/ml.

**Statistics**

All values were expressed as mean±SD. All data analysis was performed with the use of SPSS 13.0 statistical software. One-way ANOVA followed by multiple comparisons with Student–Newman–Keuls test was used to determine the effects of treatments on
the various parameters. Survival rates during follow up among the six groups were analyzed by standard Kaplan-Meier analysis, and a statistical comparison between survival curves was made with the log-rank test. Fisher’s exact method was used for nonparametric data. Statistical significance was defined as $P<0.05$.

**Supplementary results:**

**Improved survival rate by intensive postconditioning**

During the 84 day observation period, PerC, D-3PostC and D-1PostC resulted in improved survival rate compared with MI and SP groups ($P<0.05$, for all). Interestingly, the improved survival rate was apparent as early as 28 days after MI only in D-1PostC group whereas in D-3 PostC group this effect was not observed until 56 days after MI.

Furthermore, on day 84, D-1 PostC was associated with improved survival rate compared with PerC and D-3PostC treatments ($P<0.05$, respectively). Daily delivery of sodium pentobarbital did not offer any beneficial or adverse effects during the observation period compared with MI group ($P>0.05$) (Fig. I B). Autopsy in those animals who died showed no incidence of cardiac rupture in the treated groups, 1 in SP group and 1 in MI group, with no statistical significance between the different groups ($P>0.05$, respectively).

**Effect on infarct size**

Infarct size was quantified for the six different groups at seventy-two hours after reperfusion. Area at risk delineated by Evans blue was similar between the six groups
There was a significant decrease in infarct size in the remote conditioning-treated groups (PerC, 35.63±4.21%; D-3 PostC, 33.88±3.52% and D-1 PostC, 31.88±4.82%) compared with MI group (50.5±4.11%. \( P<0.05 \), respectively), with no statistical differences between the three remote conditioning treated groups \( (P>0.05, \text{respectively}) \), indicating that repetitive remote postconditioning treatment has no additional effect on infarct size over a single episode of perconditioning. In contrast, daily sodium pentobarbital administration did not affect the infarct size (46.4±2.88%) compared with that in MI group \( (P>0.05) \) (Fig. I I A&B).

The same pattern of reduction in infarct size was also observed on day 28, with a reduction in infarct size resulting from all remote conditioning therapies compared with MI group \( (P<0.05, \text{respectively}) \), but no difference between the conditioning groups \( (P>0.05, \text{respectively. Fig. I I C&D}) \).

**Attenuated Inflammatory Response at Early Phase**

Early phase inflammatory response was also evaluated on day 4 (72 hours after reperfusion), five heart tissue slices were collected from each group respectively for immunohistochemical staining. Macrophage and neutrophil infiltration into the border zone was minimal in the sham group using antibody targeting ED-1 and MPO, respectively. However, there was intensive infiltration by both macrophages and neutrophils detected in MI group rats (ED-1 cells, 1722±217/mm²; MPO cells, 880±144/mm²), which was attenuated in PerC rats \( (P<0.01, \text{respectively}) \).

Macrophage and neutrophil infiltration was further attenuated by D-3 PostC and D-1
PostC ($P<0.01$ vs. MI group, respectively) with the greatest effect detected in D-1 PostC group rats ($P<0.05$ vs. D-3 PostC group). There was no modification of inflammatory responses in the SP ($P>0.05$ vs. MI group, respectively) (Fig. I I I A-D).

The expression level of MCP-1 in the infarction border zone on day 4 showed exactly the same pattern as the macrophage and neutrophil infiltration, demonstrating a dose-dependent effect of the remote conditioning therapies on this marker of the inflammatory response ($P<0.05$, respectively. Fig. I I I E).

**Oxidative stress, NF-κB activation and cytokine expression**

MDA concentration was quantified to assess the oxidative stress. The concentration of MDA was significantly increased in MI rats on day 28, remote conditioning also resulted in a significant dosage dependent reduction in MDA accumulation, whereas daily anesthetic intervention (SP group) did not affect the MDA levels (online Fig. I A).

Consistent with MDA measurement, PerC also resulted in less phosphorylation of the NF-κB subunit p65 and its inhibitory protein IκBα ($P<0.05$ vs. MI group, respectively). Both D-3 PostC and D-1 PostC were associated with a further decrease in the degree of p65 and IκBα phosphorylation ($P<0.05$ vs. PerC group, respectively), with D-1 PostC group rats showing the lowest activation of NF-κB signaling ($P<0.05$ vs. D-3 PostC) (online Fig. I B & C).

Interestingly, on day 28 PerC did not modify TNF-α and IL-1β levels ($P>0.05$, respectively), which were only attenuated by both D-3 PostC and D-1 PostC groups ($P<0.01$ vs. MI group, respectively), however, to the same degree ($P>0.05$) (online Fig.
Fibrosis Responses

The modulation of TGF-β1/Smad2 signaling activation by remote conditioning was consistent with the beneficial effects pattern on NF-κB signaling activation (online Fig. 2A & B), and further supported by attenuated interstitial fibrosis shown in Masson trichrome (online Fig. I I C & D) and Sirius red staining (online Fig. I I E).

Comparatively, the gradient effects were not apparent when TGF-β1/Smad2 signaling activation was quantified on remote heart tissue, however, with D-1 PostC showing the greatest suppression on TGF-β1/Smad2 signaling activation (online Fig. I I A & B).

Hypertrophic Response

The increase in LVMI seen in the MI group (3.25±0.54 mg/g vs. Sham 2.08±0.34 mg/g; \( P<0.05 \)) was attenuated by PerC (2.73±0.31 mg/g; \( P<0.05 \)). A further decrease was detected in both D-3 PostC and D-1 PostC (D-3 PostC, 2.51±0.32mg/g; D-1 PostC, 2.18±0.28 mg/g, \( P<0.05 \) vs. PerC group, respectively), with the lowest LVMI observed in D-1 PostC (\( P<0.001 \), vs. D-3 PostC group).

Quantification of hypertrophy-related genes expression showed that increased gene expression of ANP and β-MHC was attenuated while the decrease in expression of α-MHC was recovered by remote conditioning, again in a dosage dependent
fashion (online Fig. IV A-C). However, the effects were absent when only sodium pentobarbital was given daily.

**Improved Cardiac Geometry, Function and Hemodynamic Parameters**

On day 28, MI rats demonstrated significant LV dilation, as evidenced by increased LVEDD compared with sham rats ($P<0.05$). This geometric change was accompanied by decreased FS compared with sham rats ($P<0.05$). While PerC significantly improved adverse LV remodeling, reflected by a decrease in LVEDD and an increase in FS compared with MI rats ($P<0.05$, respectively), repeated remote PostC therapy resulted in a further improvement in LV chamber size and function, with the greatest effects achieved by D-1 PostC in comparison with D-3 PostC ($P<0.05$, respectively) (Fig. IV A-D). Hemodynamic analysis demonstrated the same pattern of benefits from remote conditioning, in a dose-dependent manner compared with the MI group (online Fig. V A-C). Again, none of these beneficial effects was observed in SP group.

**Online supplement references**


Supplementary Figures

Online figure 1. Panel A: Bar graph demonstrated different extent of MDA accumulation in myocardium at the border zone of different groups. Representative western blot bands showed phosphorylation level of NFkB P65 (Panel B) and its inhibitory protein IκBα (Panel C) with quantification shown in bar graphs below for peri-infarct heart tissue obtained from each group rats (GAPDH as loading control). Quantitative analysis of plasma TNF-α (Panel D) and IL-1β (Panel E) levels were measured for each group rats on day 28 after MI. Data are expressed as mean ± SD, with the same abbreviations as in text.
Online figure I I. Representative Western blot bands showed TGF-β1 expression (Panel A) and phosphorylated Smad2 over total Smad2 protein expression (Panel B), with protein quantification for peri-infarcted heart tissue obtained from each group shown in bar graphs below (adjusted by GAPDH expression). Panel C&D demonstrated representative sections with Masson’s trichrome staining (fibrosis stained in blue, magnification ×400, scale bar =50 μm) within the border zone of hearts and its quantification shown in bar graph for each group. Panel E: Sirius red staining further confirmed the same pattern of fibrosis for each group as Masson’s trichrome staining. Data are expressed as mean±SD, with the same abbreviations as in text.
Online figure I I I. Representative Western blot bands showed TGF-β1 expression (Panel A) and phosphorylated Smad2 over total Smad2 protein expression (Panel B), with protein quantification for remote 'normal' heart tissue obtained from each group shown in bar graphs below (adjusted by GAPDH expression).
Online figure IV. Bar graphs showing the mRNA expression quantification of ANP (Panel A), α-MHC (Panel B), and β-MHC (Panel C), respectively. Data are expressed as mean±SD, with the same abbreviations as in text.
Online figure V. Hemodynamic data including LVEDP (Panel A), dP/dt\textsubscript{max} (Panel B) and dP/dt\textsubscript{min} (Panel C) were quantified. Data are expressed as mean±SD, with the same abbreviations as in text.